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Demonstration of Sustained Drug-Resistant Human Immunodeficiency Virus Type 1 Lineages Circulating among Treatment-Naïve Individuals▽†

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Transmission of human immunodeficiency virus (HIV) drug resistance is well-recognized and compromises response to first-line therapy. However, the population dynamics of transmitted resistance remains unclear, although previous models have assumed that such transmission reflects direct infection from treated individuals. We investigated whether population-based phylogenetic analyses would uncover lineages of resistant viruses circulating in untreated individuals. Through the phylogenetic analysis of 14,061 HIV type 1 (HIV-1) *pol* gene sequences generated in the United Kingdom from both treatment-naïve and -experienced individuals, we identified five treatment-independent viral clusters containing mutations conferring cross-resistance to antiretroviral drugs prescribed today in the United Kingdom. These viral lineages represent sustainable reservoirs of resistance among new HIV infections, independent of treatment. Dated phylogenies reconstructed through Bayesian Markov chain Monte Carlo inference indicated that these reservoirs originated between 1997 and 2003 and have persisted in the HIV-infected population for up to 8 years. Since our cohort does not represent all infected individuals within the United Kingdom, our results are likely to underestimate the number and size of the resistant reservoirs circulating among drug-naïve patients. The existence of sustained reservoirs of resistance in the absence of treatment has the capacity to threaten the long-term efficacy of antiretroviral therapy and suggests there is a limit to the decline of transmitted drug resistance. Given the current decrease in resistance transmitted from treated individuals, a greater proportion of resistance is likely to come from drug-naïve lineages. These findings provide new insights for the planning and management of treatment programs in resource-rich and developing countries.

The introduction of highly active antiretroviral therapy in the mid-1990s marked the most significant advance in the management of human immunodeficiency virus (HIV) infection. There are currently more than 20 drugs available for use against HIV, targeting five different aspects of viral replication, including reverse transcriptase (RT), protease (PR), and integrase activities (10, 13, 37, 39, 46). When used in combination, these drugs suppress HIV replication, leading to clinical benefit (9, 28, 29). Nevertheless, drug-resistant viruses can emerge and have been documented in patients treated with every known class of drugs (22, 27, 34). The fixation of drug resistance mutations (DRMs) in an HIV population results from the evolutionary competition between genetic variants (38). Because of the selective advantage these mutations confer in the presence of drugs, their rate of fixation is fast. Within weeks of starting treatment, drug-resistant mutants can predominate in the plasma viral RNA (40).

Between 50 and 70% of treated patients with virological rebound harbor some form of drug-resistant virus (17). This has two consequences. First, some resistance-associated mutations cause cross-resistance to other drugs within the same class and future drug options become limited (19). Second, since high levels of plasma viremia are associated with infectivity (32, 35) these mutants can be transmitted to other patients (4, 33, 44). Transmitted drug resistance has reached between 5 and 10% in areas of the world with access to treatment (41, 45, 51), compromising response to first-line therapy (25). It is assumed that such transmission reflects direct infection from drug-experienced individuals. It follows that recent improvements in the treatment of HIV infections, with higher rates of viral suppression (31), will lead to reductions in transmitted resistance. Indeed, such reductions have been recently reported (45).

The extent to which acquired resistance persists in the infected population is unclear. It is generally accepted that most DRMs are associated with a fitness cost (11, 16). Thus, wild-type viruses commonly reemerge from archived reservoirs in treated patients who stop therapy following the emergence of drug resistance (7, 21). By contrast, transmitted resistance appears to be more long-lived in plasma virus even in the absence of treatment (24). Since the probability for a mutation to be transmitted is positively correlated with its persistence in a viral population, resistant polymorphisms fixed during the early stage of the disease have an increased chance to spread within the community. This can lead to the establishment of reservoirs of resistance among new HIV infections, indepen-

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dent of treatment. As up to 20 to 40% of infected individuals remain undiagnosed in the United Kingdom (18) and transmission events often occur around the time of primary infection, long before antiretroviral therapy is instigated (32), the potential for such reservoirs is a major concern.

In addition to traditional epidemiological approaches, phylogenetic analyses of viral gene sequences have substantially broadened our understanding of the role played by newly infected individuals in HIV epidemics (1, 15, 23, 30). Importantly, the contribution of untreated patients to the spread of antiretroviral resistance has not been thoroughly addressed. Moreover, the extent to which reservoirs of resistant HIV mutants may persist in a drug-naïve population in the absence of treatment has yet to be defined.

Here, we sought to identify potential lineages of resistant viruses circulating in drug-naïve patients, as evidence of treatment-independent reservoirs of resistance. Through the phylogenetic analysis of the largest United Kingdom database of HIV-1 sequences, we have identified drug-naïve resistant HIV-1 strains and characterized the population dynamics of these lineages. The demonstration of such a phenomenon raises concern for long-term effectiveness of antiretroviral therapies, both in resource-rich countries and in those areas of the world now rolling out therapy to those in need.

MATERIALS AND METHODS

Study population. The 14,061 HIV-1 *pol* gene sequences analyzed here were extracted from the UK HIV Drug Resistance Database (2). This database is estimated to hold over 90% of all such sequences amplified within the United Kingdom. The sequences were generated through routine drug resistance testing between 1997 and 2006 on individuals with known treatment status. When multiple samples were available for a drug-naïve patient, only baseline samples were selected for the analysis, while samples from the most recent time point were retained from drug-experienced individuals. This gave a total of 7,705 (55%) and 6,356 (45%) viral sequences from drug-naïve and -experienced individuals, respectively. The sequences were coded for anonymity, but information on geographical region, self-reported route of transmission, and date of sampling was retained. All main exposure groups were represented in the cohort: men having sex with men (MSM; $n = 5,397$), heterosexual transmission ($n = 2,290$), injection drug users ($n = 274$), those in contact with blood products ($n = 44$), and other exposure groups ($n = 11$). The route of transmission for 6,045 patients was not documented.

The sequences were generated in clinical virology and commercial laboratories serving United Kingdom clinical centers. The sequences used included the entire protease region (297 bp) and a partial reverse transcriptase region (1,023 bp) of the HIV genome. Subtypes and resistance profiles were established by electronic submission to the Rega subtyping algorithm (6) and the Stanford HIV Drug Resistance Database (http://cpr.stanford.edu/). The cohort included sequences of subtypes A ($n = 788$), B ($n = 8,850$), C ($n = 2,720$), D ($n = 233$), F ($n = 42$), G ($n = 189$), CRF01_AE ($n = 157$), and CRF02_AG ($n = 355$), as well as complex recombinant forms ($n = 727$).

Phylogenetic reconstruction and resistance mapping. The sequences were divided per subtype and manually aligned using the sequence editor Se-Al v2.0 (36). Because of the size of the data set, the phylogenies of clade A, B, and C sequences were reconstructed by neighbor-joining inference, under the General Time Reversible model of nucleotide substitution with proportion of invariable sites and gamma-distributed rate heterogeneity (GTR + I + Γ). For smaller data sets (i.e., subtypes D, F, G, CRF01_AE, and CRF02_AG), maximum likelihood trees were inferred under a similar model. All phylogenies were reconstructed using the software Paup* 4.0 beta 10 (43).

In order to identify drug resistance mutations sharing common ancestry in the data set, the ancestral states of resistance-associated codon positions were reconstructed at the internal nodes of the trees. A total of 37 codons (30, 32, 33, 46, 47, 48, 50, 54, 76, 82, 84, 88, and 90 in PR, and 41, 62, 65, 67, 70, 74, 75, 77, 100, 103, 106, 108, 115, 116, 151, 181, 184, 188, 190, 210, 215, 219, 225, and 236 in RT) were investigated, according to the International AIDS Society USA Drug Resistance Mutations Group guidelines (http://www.iasusa.org). These were positions known to be associated with high-level or primary resistance to protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs), and nonnucleoside reverse transcriptase inhibitors (NNRTIs). An initial ancestral reconstruction was performed using a maximum parsimony approach (12) with the software McClade version 4.07 (26). Parsimony reconstruction methods determine the ancestral state that minimizes the number of mutational changes along a phylogenetic tree, given an observed distribution of characters (here, the sequence alignment).

To characterize virus lineages harboring transmitted drug resistance mutations in epidemiologically linked drug-naïve individuals, we looked for resistance-associated polymorphisms present at internal nodes that linked patients with no known treatment history. For each tree, the phylogenetic clustering of three or more sequences from drug-naïve patients sharing at least one resistance-associated polymorphism was considered evidence for the existence of a drug-resistant viral lineage.

Confirmation of resistant viral lineages. When potential drug-naïve resistant lineages were identified, the reliability of the clusters was confirmed on smaller data sets by using more robust phylogenetic methods. First, the sequences of interest were compared to a set of 100 random sequences from either treated or untreated patients using Bayesian Markov chain Monte Carlo (MCMC) inference. Bayesian posterior trees were generated with the software BEAST version 1.4.6 (8), under the GTR + Γ model of evolution and assuming a relaxed molecular clock. The MCMC search was set to 100,000,000 iterations and the trees were sampled every 1,000th generation after a 10% burn-in. The maximum clade credibility tree was selected from the sampled posterior trees with the program TreeAnnotator (http://beast.bio.ed.ac.uk/).

Second, the effect of convergent evolution on our phylogenetic reconstructions was assessed by repeating the analyses (i) after removing from the alignment 59 codon positions known to be associated with drug resistance (http://www.iasusa.org), leaving 1,143 bp, and (ii) taking into account third-codon positions only (440 nucleotides). The MCMC search was set to 100,000,000 iterations, and trees were sampled every 1,000th generation after a 10% burn-in. Each cluster was also independently compared to the 100 sequences most closely related to those of interest in the original neighbor-joining tree, and their respective robustness was assessed by bootstrap analyses under the two conditions listed above. Bootstrap scores were obtained on the basis of 1,000 neighbor-joining replicates.

Finally, the reconstruction of the clusters' ancestral states was confirmed by maximum likelihood reconstruction, using the marginal reconstruction approach implemented by the program codeml of the PaML package version 3.14a (49). This approach compares the probabilities of different character assignments to an internal node at a site and selects the character that has the highest posterior probability (50). Maximum likelihood reconstructions were performed under the Whelan and Goldman empirical model of protein evolution (48).

Timing of the emergence of drug-resistant lineages. The time of the most recent common ancestor of each drug-resistant lineage, corresponding to the date at which resistance mutations occurred in the clusters, was estimated using the Bayesian MCMC approach implemented in BEAST. For computational reasons, the data were downsized to 100 sequences isolated between 1997 and 2006 from both drug-naïve and -experienced patients. Estimates were sampled every 1,000th generation from an MCMC search of 10,000,000 iterations under two different molecular clock models: a strict molecular clock model and an uncorrelated lognormal relaxed clock model. Bayesian skyline plots were used as coalescent tree priors. The model with the best fit to the data was used for interpretation, as determined by the calculation of the models' Bayes factor (BF). The Bayes factor corresponds to the ratio of the marginal likelihood of the models tested, and a difference in the BF above 20 strongly supports the favored model. The BF of the models was calculated using a script available at http://code.google.com/p/beast-mcmc/ (42).

Nucleotide sequence accession numbers. The sequences used in the study were registered with GenBank under the accession numbers EU817047 to EU817091.

RESULTS

Identification of antiretroviral drug-resistant HIV-1 lineages. Phylogenetic trees were built for each HIV subtype present in the cohort (phylogenies available on request). The nucleotide sequences of viral ancestors lying at each of the inter-

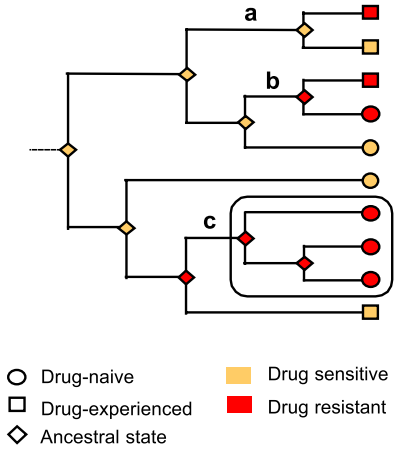

FIG. 1. Schematic distribution of DRMs found in the reconstructed HIV-1 *pol* gene phylogenies. Circles and squares at the tips of the branches represent drug-naïve and -experienced patients, respectively. Sequences with drug resistance mutations are indicated in red. Three distribution patterns were distinguished: sporadic acquisition of DRMs (a), treatment-dependent transmission of DRMs (b), and treatment-independent transmission of DRMs (c). Lineages of drug-resistant viruses circulating among newly infected individuals were defined as clusters of resistant viruses that included three or more drug-naïve individuals (c).

nal nodes of the trees were reconstructed in order to identify resistance mutations sharing a common descent. A mutation acquired in a sampled patient would be positioned on the terminal branch leading to the corresponding virus and expected to share no ancestry with adjacent sequences in the phylogeny. This is generally the case for resistance mutations arising through drug-induced selective pressure. Conversely, a polymorphism fixed prior to transmission will be shared by more than one adjacent virus in the tree and will be present at the node linking the isolates. Three patterns of resistance mutation distributions were seen within the phylogenies: (i) sporadic DRMs, (ii) treatment-dependent transmitted DRMs, and (iii) treatment-independent transmitted DRMs. A schematic representation of these three types of DRM distributions is shown in Fig. 1. Sporadic DRMs (i.e., mutations that did not share common ancestry with other DRMs in the tree) (Fig. 1a) were noted in all clades. These represent individual adaptations to treatment when observed in drug-experienced patients or infection from an unsampled drug-experienced source when in a drug-naïve individual. Treatment-dependent transmitted DRMs consisted of phylogenetically linked resistance mutations shared either by multiple drug-experienced individuals or by a combination of drug-experienced and -naïve patients. These represent clusters of resistant viruses probably transmitted from treated to untreated individuals (Fig. 1b). Finally, treatment-independent transmitted DRMs were identified as resistance mutations circulating within phylogenetically linked drug-naïve patients only (Fig. 1c). Phylogenetic clusters including the latter type of DRM distribution were thought to represent drug-resistant HIV-1 populations circulating among untreated individuals.

No clusters of more than three drug-naïve patients were found to harbor resistance mutations in subtypes other than B. However, five treatment-independent drug-resistant lineages were identified within the clade B data set. The initial neighbor-joining tree of 8,850 clade B sequences is too large to be reproduced here. Therefore, the topologies to each of the individual clusters extracted from Bayesian phylogenies are shown in Fig. 2. The clusters, labeled A to E, included 19, 9,

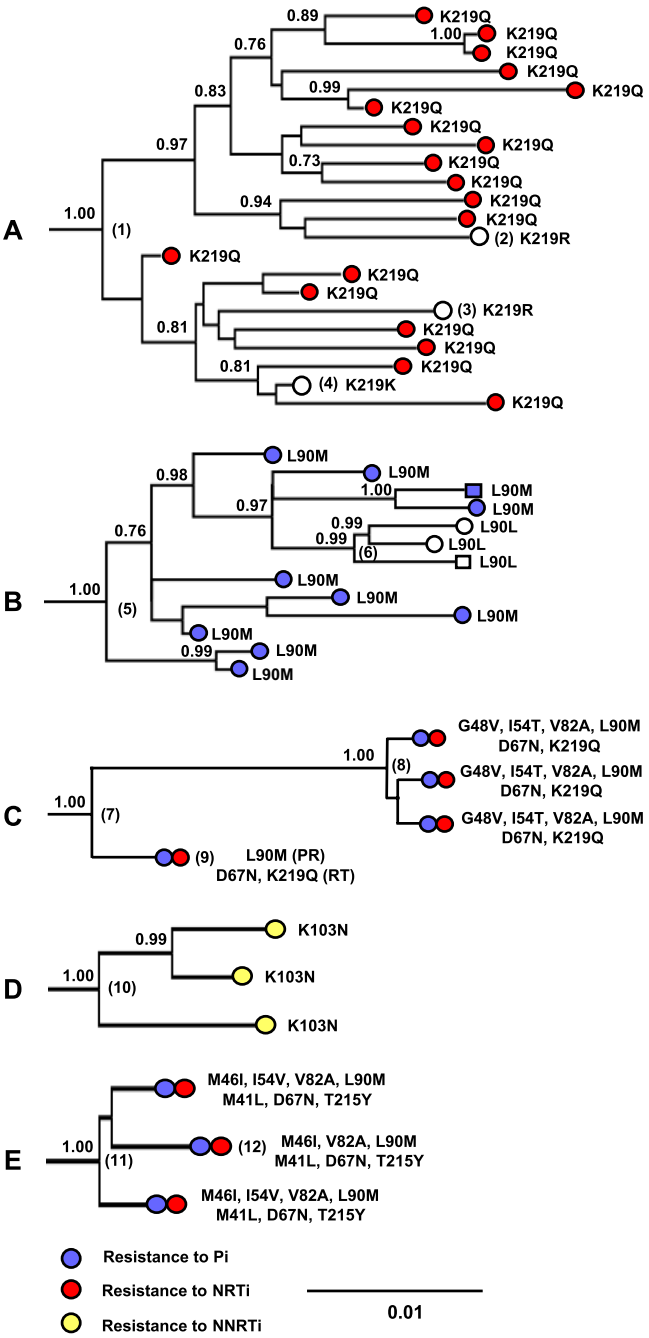

FIG. 2. Phylogenies of the five resistant HIV-1 clusters circulating among drug-naïve individuals. The clusters were extracted from a Bayesian MCMC maximum clade credibility tree generated under the GTR + Γ model of evolution and a relaxed molecular clock. Branch lengths are expressed in nucleotide substitutions per site, and Bayesian posterior probabilities are shown when above 0.5. Terminal branches are labeled with the resistance mutations found in the respective sequences. Circles and squares symbolize drug-naïve and -experienced patients, respectively. Labels at internal nodes or tips of the trees correspond to the branches listed in Table 2.

TABLE 1. Details of the five treatment-independent drug-resistant lineages circulating in the United Kingdom

Cluster	Sequence	Sampling date	Risk group	Shared drug resistance mutation(s)			Atypical RT mutation	Treatment history	Accession no.
				PIs	NRTIs	NNRTIs			
A	s1	2005	MSM		K219Q		S134C	Naïve	EU817047
A	s2	2005	MSM		K219Q		S134C	Naïve	EU817058
A	s3	2001	MSM				S134C	Naïve	EU817062
A	s4	2005	MSM		K219Q		S134C	Naïve	EU817063
A	s5	2005	Unknown		K219Q		S134C	Naïve	EU817064
A	s6	2005	MSM		K219Q		S134C	Naïve	EU817065
A	s7	2003	Unknown				S134C	Naïve	EU817066
A	s8	2004	Unknown		K219Q		S134C	Naïve	EU817067
A	s9	2005	MSM		K219Q		S134C	Naïve	EU817068
A	s10	2004	Heterosexual				S134C	Naïve	EU817048
A	s11	1998	MSM		K219Q		S134C	Naïve	EU817049
A	s12	2003	MSM		K219Q		S134C	Naïve	EU817050
A	s13	2005	MSM		K219Q		S134C	Naïve	EU817051
A	s14	2005	Unknown		K219Q		S134C	Naïve	EU817052
A	s15	2003	Unknown		K219Q		S134C	Naïve	EU817053
A	s16	2004	Unknown		K219Q		S134C	Naïve	EU817054
A	s17	2005	Unknown		K219Q		S134C	Naïve	EU817055
A	s18	2005	MSM		K219Q		S134C	Naïve	EU817056
A	s19	2003	Unknown		K219Q		S134C	Naïve	EU817057
A	s20	2001	MSM		K219Q		S134C	Naïve	EU817059
A	s21	2005	MSM		K219Q		S134C	Naïve	EU817060
A	s22	2004	MSM		K219Q		S134C	Naïve	EU817061
B	s23	2005	MSM					Experienced	EU817069
B	s24	2005	MSM	L90M				Experienced	EU817070
B	s25	2000	MSM	L90M				Naïve	EU817071
B	s26	2002	MSM	L90M				Naïve	EU817072
B	s27	2005	Unknown					Naïve	EU817073
B	s28	2000	MSM	L90M				Naïve	EU817074
B	s29	2004	MSM					Naïve	EU817075
B	s30	2005	Unknown	L90M				Naïve	EU817076
B	s31	2005	MSM	L90M				Naïve	EU817077
B	s32	2004	MSM	L90M				Naïve	EU817078
B	s33	2000	MSM	L90M				Naïve	EU817079
B	s34	2003	MSM	L90M				Naïve	EU817080
B	s35	2001	MSM	L90M				Naïve	EU817081
C	s36	2002	MSM	L90M	D67N, K219Q			Naïve	EU817085
C	s37	2005	MSM	G48V, I54T, V82A, L90M	D67N, K219Q			Naïve	EU817083
C	s38	2005	MSM	G48V, I54T, V82A, L90M	D67N, K219Q			Naïve	EU817084
C	s39	2005	Unknown	G48V, I54T, V82A, L90M	D67N, K219Q			Naïve	EU817082
D	s40	2005	Unknown			K103N		Naïve	EU817086
D	s41	2005	Unknown			K103N		Naïve	EU817087
D	s42	2005	Unknown			K103N		Naïve	EU817088
E	s43	2005	Unknown	M46I, V82A, L90M	M41L, D67N, T215Y		T4P, K103S	Naïve	EU817090
E	s44	2005	MSM	M46I, I54V, V82A, L90M	M41L, D67N, T215Y		T4P, K103S	Naïve	EU817089
E	s45	2005	Unknown	M46I, I54V, V82A, L90M	M41L, D67N, T215Y		T4P, K103S	Naïve	EU817091

4, 3, and 3 patients, respectively. The branch supporting each cluster showed a Bayesian posterior probability of 1.00, indicating maximal support for their existence. Details of the five treatment-independent drug-resistant lineages are given in Table 1.

Confirmation of the resistant viral lineages. The genetic relatedness of the viruses found in the clusters was confirmed by Bayesian MCMC inference and bootstrap analyses under two conditions: (i) after excluding from the alignment 59 drug resistance-associated sites to circumvent the effect of drug-induced convergent evolution and (ii) based on third-codon positions only, to rule out convergent evolution unassociated with drug pressure. The five resistant clusters were consistently present in the Bayesian maximum clade credibility trees generated under the above conditions and supported by a posterior probability of 1.00 (Fig. 3). The five lineages were also robustly supported by a bootstrap score of >95% when individually compared to the 100 sequences most closely related to those of interest in the original neighbor-joining tree (data not shown). This remained true for bootstrapped trees constructed on the basis of these alignments. Our findings exclude the possibility of artifactual phylogenetic clustering induced by convergent evolution. Maximum likelihood ancestral reconstructions confirmed that the DRMs found in the lineages were

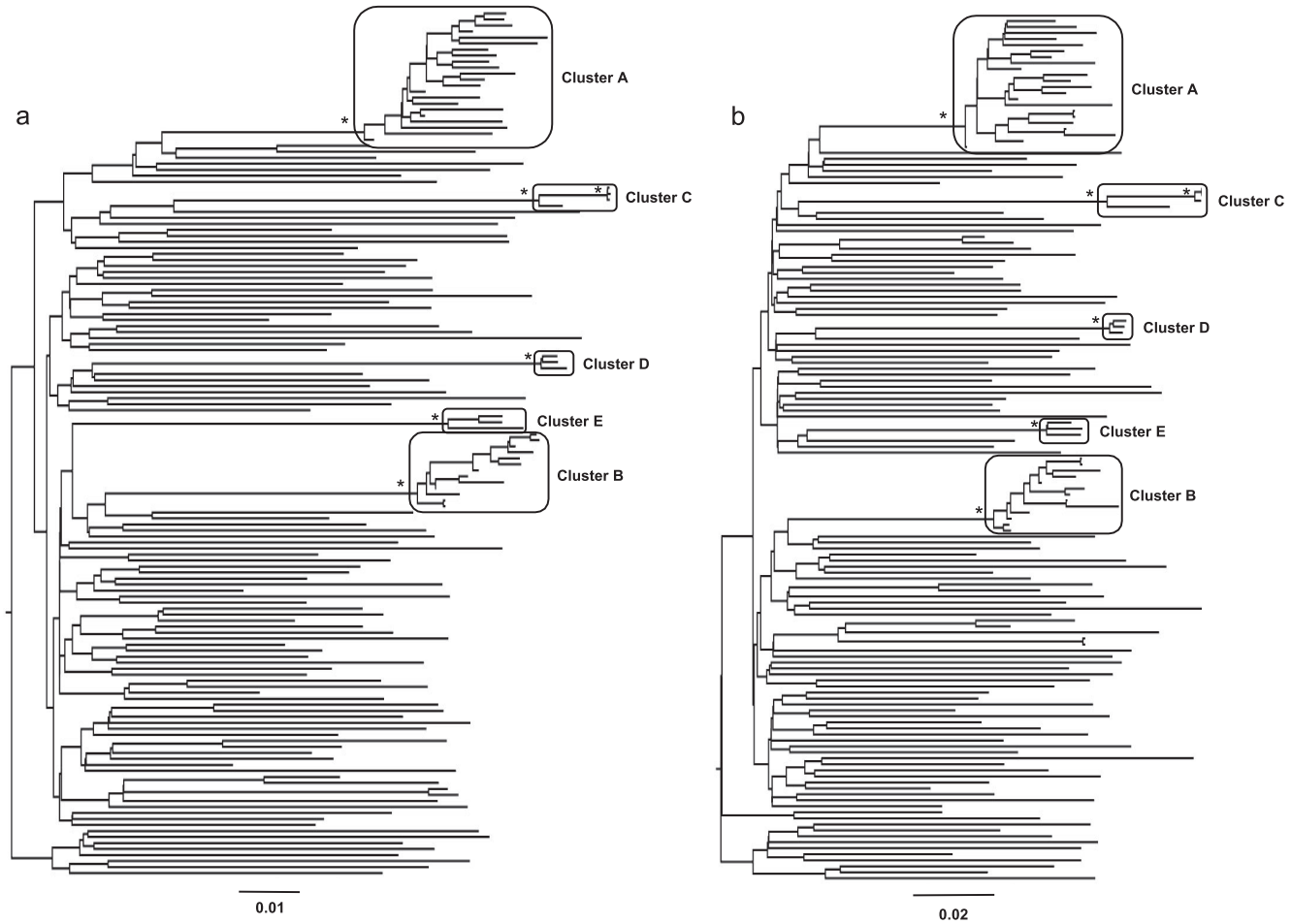

FIG. 3. Bayesian MCMC phylogenetic trees of the resistant lineages compared to 100 HIV-1 *pol* sequences after exclusion of 59 codon positions associated with drug resistance (a) or based on third-codon positions only (b). Branch lengths are expressed as the number of nucleotide substitutions per site. Boxes indicate the position of the five resistant clusters in the trees. Nodes with a posterior probability of 1.00 are labeled with a star.

fixed prior to the infection of the individuals involved in the transmission clusters (Table 2).

Description of the treatment-independent, drug-resistant lineages. Clusters A, B, and D harbored mutations conferring reduced susceptibility to drugs within a single class of antiretrovirals, while the two others exhibited genotypic resistance to more than one class of drugs. Resistance to PIs, NRTIs, and NNRTIs were found in three, three, and two clusters, respectively. Clusters A and E also included viruses with atypical mutations otherwise absent in the entire data set (S134C in RT for cluster A; T4P in PR and K103S in RT for cluster E), confirming further the common ancestry of these viruses. Patients in the clusters for which the exposure group was known (29 of 45) were all men having sex with men, apart from one patient who reported heterosexual contact as the route of infection. When known, the geographical locations of the patients involved in the clusters corroborated the transmission networks. Patients from clusters A, B, and C, for instance, attended clinics in the greater London area, while cluster D involved patients from the East Midlands.

Cluster A included viruses sampled from 22 drug-naïve patients between 1998 and 2005, 19 of whom harbored viruses with the K219Q resistance genotype in RT. One virus of the cluster exhibited a drug-sensitive genotype at position 219 (i.e., s3), while the replacement of a glycine with an arginine (CAA to CGA) at the same position led to the independent loss of the resistance genotype in two patients (s7 and s10) (Table 2). Cluster B included 13 viruses sampled between 2000 and 2005. Two of the patients (s23 and s24) had experienced antiretroviral treatment. This lineage also showed evidence of sporadic reversions to wild type. The L90M resistance genotype was lost along an internal branch of the cluster by the effect of a single nucleotide substitution (ATC to CTG) (Table 2) before transmission to three individuals (Fig. 2). Three of the four DRMs found in cluster C (i.e., G48V, I54T, and V82A in PR) (Table 1) were absent in the earliest sequence of the cluster. The branch separating this subcluster from the oldest sample of the lineage (i.e., s36) is suggestive of a longer interval between transmission events or of transmission through individuals not present in the data set. Maximum likelihood ancestral reconstructions indicated that the G48V, I54T, and V82A mutations reached fixation during that time period (Table 2), either following the treatment of patient s36 before transmission to the other patients or in treated individuals missing from the co-

TABLE 2. Maximum likelihood reconstruction of the five lineages' ancestral states at drug-resistant codon positions

Cluster	Branch[a]	Codon position (product)	Codon (encoded amino acid)	Probability[b]	Altered codon[c] (encoded amino acid)	Probability[b]
A	1	219 (RT)	AAA (K)	0.97	**C**AA (Q)	1.00
	2	219 (RT)	CAA (Q)	1.00	C**G**A (R)	
	3	219 (RT)	CAA (Q)	1.00	C**G**A (R)	
	4	219 (RT)	CAA (Q)	1.00	**A**AA (K)	
B	5	90 (PR)	TTG (L)	0.96	**A**TG (M)	0.99
	6	90 (PR)	ATG (M)	1.00	**C**TG (L)	1.00
C	7	90 (PR)	TTG (L)	0.98	**A**TG (M)	0.96
		82 (PR)	GTC (V)	0.99	G**C**C (A)	0.99
		67 (RT)	GAC (D)	0.99	**A**AC (N)	0.92
		219 (RT)	CAA (Q)	1.00	C**G**A (R)	1.00
	8	48 (PR)	GGG (G)	0.99	G**T**G (V)	1.00
		54 (PR)	ATC (I)	0.99	A**C**C (T)	1.00
	9	54 (PR)	ATC (I)	0.99	**G**TC (V)	
		82 (PR)	GCC (A)	0.99	**A**CC (T)	
D	10	103 (RT)	AAA (K)	0.99	AA**C** (N)	1.00
E	11	46 (PR)	ATG (M)	1.00	AT**A** (I)	1.00
		54 (PR)	ATC (I)	0.99	**G**TC (V)	0.97
		82 (PR)	GTC (V)	1.00	G**C**C (A)	1.00
		90 (PR)	TTG (L)	0.81	**A**TG (M)	1.00
		67 (RT)	GAC (D)	0.99	**A**AC (N)	1.00
		41 (RT)	ATG (M)	1.00	**T**TG (L)	1.00
		215 (RT)	TTC (T)	1.00	TA**C** (Y)	1.00
	12	54 (PR)	GTC (V)	0.97	**A**TC (I)	

[a] Branch numbers correspond with those shown in Fig. 2.
[b] Posterior probability of the assigned character at the nodes linked by the given branch (see reference 49).
[c] The altered nucleotide is shown in boldface.

hort. In contrast, the DRMs conferring resistance to NRTIs (D67N and K219Q in RT) were common to all four patients, indicating a more ancient fixation in the lineage. Clusters D and E represented the smallest clusters identified, each involving three patients sampled during the year 2005. Cluster E also showed evidence of reversion from drug resistance to wild type. The I54V mutation was absent in the viruses from patient s43, while it was found in the two adjacent isolates (Fig. 2).

All five clusters also harbored secondary or accessory mutations associated with resistance to either protease or reverse transcriptase inhibitors (see Table S1 in the supplemental material). Secondary mutations are protease polymorphisms of varying frequency that, by themselves, may not have a significant impact on phenotype but rather improve the replicative fitness of resistant viruses (19). Such polymorphisms were found in the three clusters showing genotypic resistance to protease inhibitors, i.e., clusters B, C, and E. Likewise, accessory mutations associated with high-level resistance to reverse transcriptase inhibitors (3) were found in the four clusters with resistance to the latter class of drugs (i.e., clusters A, C, D, and E). It is possible that these mutations allow key drug resistance changes to persist with limited fitness cost.

Timing of the emergence of drug-resistant lineages. The time of origin of the resistant lineages was determined using a Bayesian MCMC approach under strict and relaxed molecular clock models of evolution. The Bayes factors for the two models strongly supported the relaxed clock over the strict model (log BF, 102.59), indicating a significantly better fit of the estimates obtained under the former model. Assuming a relaxed molecular clock, the viral gene under study has evolved at a rate of 2.6×10^{-3} substitutions/site/year (95% confidence interval, 1.9×10^{-3} to 3.3×10^{-3}), with a coefficient of variation in substitution rate of 0.31 (95% confidence interval, 0.25 to 0.37). Estimates of the most recent dates at which all DRMs found in the clusters were fixed in the lineages are shown in Fig. 4a. According to these estimates, the five lineages originated between 1997 (95% highest posterior density, 1995 to 1998 [cluster A]) and 2003 (95% highest probability distribution, 2001 to 2004 [cluster E]). Clusters B, C, and D have persisted since 1998 (95% highest posterior density, 1997 to 1999), 1999 (95% highest posterior density, 1997 to 2001), and 2001 (95% highest posterior density, 1998 to 2003), respectively. The dated phylogenies of the lineages obtained under the relaxed clock model of evolution are shown in Fig. 4b. These indicate that the resistant genotypes found in the lineages have individually persisted in the United Kingdom population for an average of 5 years. The resistant lineage represented by cluster A was the oldest and has been circulating in the United Kingdom for about 8 years. Since the viral gene sequences used for the analysis represent a single patient, the internal nodes of the phylogenies reflect at least one transmission event. The internal structure of the clusters scaled in calendar years is therefore informative of the transmission histories of the clusters (23). Clusters A and B exhibit an even distribution of transmission events (i.e., of internal nodes) over 8 and 7 years, respectively. By contrast, cluster C shows a contemporary expansion of the transmission network it represents, with three out of four individuals being infected in 2004.

a

Estimated time of origin of the five resistant lineages

Cluster	Root height*	95% HPD** lower	95% HPD upper	Model
A	1997.0	1995.7	1998.0	Relaxed clock
B	1998.3	1997.1	1999.4	Relaxed clock
C	1999.7	1997.4	2001.5	Relaxed clock
D	2001.2	1998.8	2003.2	Relaxed clock
E	2003.1	2001.8	2004.4	Relaxed clock

* Time of origin of the root of the cluster
** Highest posterior density

b

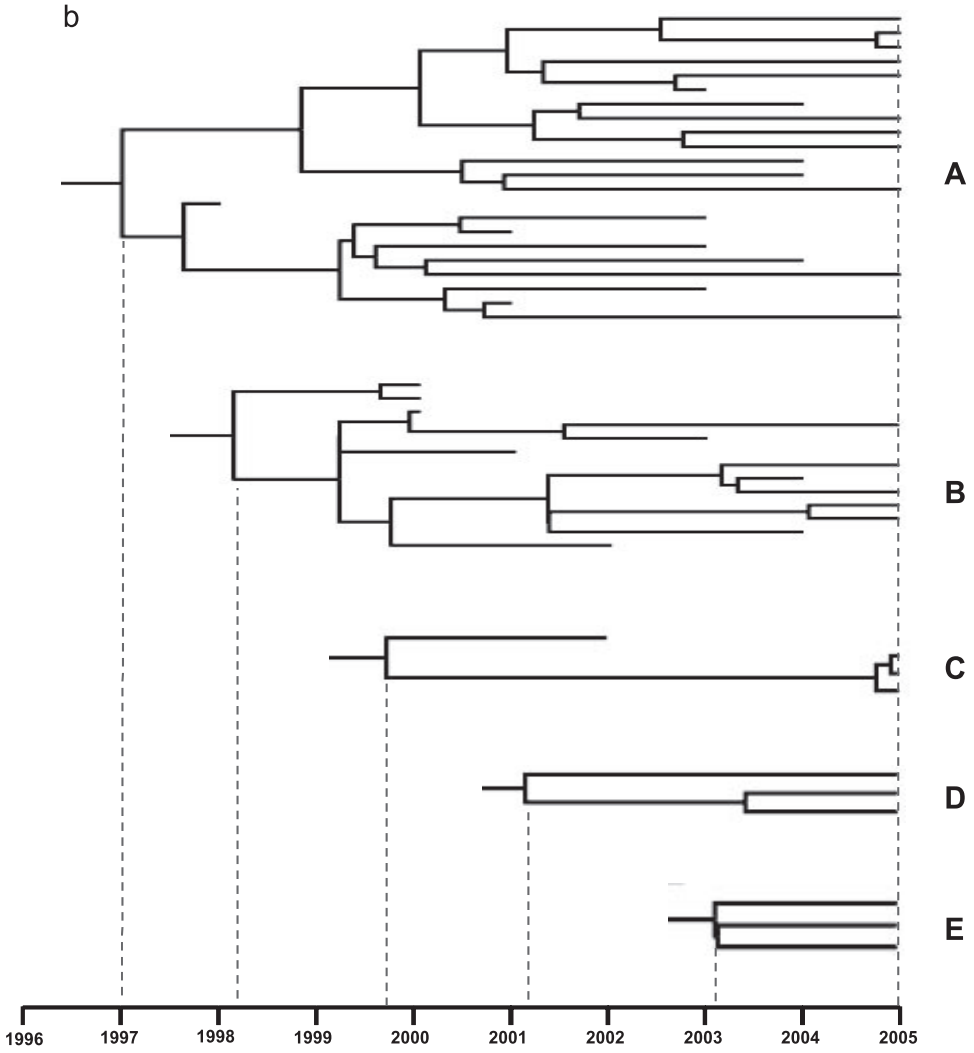

FIG. 4. (a) Estimated time of origin of the five resistant clusters circulating among drug-naïve individuals. The time of the most recent common ancestor of each cluster was estimated under a relaxed molecular clock model. (b) Relaxed clock dated phylogenies of the five resistant clusters circulating among drug-naïve individuals. Branch lengths are expressed in calendar years.

Likewise, the three patients of cluster E seem to have been infected within a short period of time during the year 2003.

DISCUSSION

Our study provides the first clear evidence of resistant HIV-1 lineages circulating among drug-naïve individuals. We believe that these lineages are indicative of treatment-independent reservoirs of resistance and represent a potential long-term risk to the continued success of antiretroviral therapy. The five independent reservoirs identified here contain mutations contributing to reduced susceptibility to drugs of the three main classes of antiretrovirals, with the largest such lineage persisting for up to 8 years. Although some mutants identified (i.e., K219Q) may not significantly change drug susceptibility on their own, their presence will lower the threshold

for subsequent emergence of resistance, and thus they represent a concern for the population efficacy of antiretroviral therapy.

The resistant lineages involved HIV subtype B viruses exclusively. The absence of non-B resistant clusters is likely due to the low number of non-B sequences available in our cohort (37%). More importantly, a majority of non-B HIV infections diagnosed in the United Kingdom were acquired abroad and subsequently introduced to the country (18), reducing the likelihood of United Kingdom-based transmission chains. However, changes in the epidemiological structure of HIV epidemics can be expected in the future, as illustrated by the recent identification of HIV transmission networks of subtypes other than B among men who have sex with men (15). The increasing availability of molecular data from imported HIV strains is likely to further establish the role of the emergent HIV diversity, especially with regard to transmitted resistance.

The survival of resistant lineages within untreated populations for up to 8 years raises questions about the persistence and transmissibility of drug-resistant variants. The fitness costs of some drug resistance mutations have been well-established (11, 16), and the presence of common polymorphic changes and/or acquisition of compensatory mutations known to increase the replicative fitness of resistant viruses is likely to have contributed to the persistence of the clusters. However, the relationship between in vitro measurement and in vivo replication is unclear, and some transmitted drug-resistant species can persist for months or years (24). Since HIV populations experience a substantial loss of genetic diversity during transmission (20), the emergence of drug-sensitive viruses in patients infected with drug-resistant strains is likely to be due to mutational reversion rather than outgrowth of archived wild-type viruses. In this regard, it is noteworthy that two of the five clusters harbor multiple resistance mutations, which may make reversion more difficult. T215Y mutations, for instance, are known to confer severe fitness cost (16) and were found in cluster E in association with other resistant polymorphisms. Yet this cluster persisted for over a year in a drug-free environment. This is suggestive of a maintained fitness in the context of other resistance mutations, similar to the compensatory mechanism identified in viruses with multiple PI resistance (47). Of the other clusters, K219Q and K103N are not thought to confer major fitness loss in isolation (5, 47). The persistence of such drug-resistant viruses in drug-naïve patients could also result from short transmission intervals, such that resistance polymorphisms are transmitted to the next individual before reversion occurs.

The dated phylogenies reconstructed in the present study provide new insights into the temporal structure of resistance transmission among untreated HIV patients. According to our estimates, three of the five resistant lineages originated between 1999 and 2003, which corresponds to the time when the transmission of DRMs was at its highest in the United Kingdom (45). The distribution of transmission patterns in the large clusters, such as clusters A and B, clearly indicates that infections among drug-naïve individuals have taken place over several years, reinforcing the idea of transmission networks involving more than one individual. However, the hypothesis that a single transmitter infected more than one individual cannot be totally excluded for cluster E, where the dates of transmission events fell within a narrow time frame.

The existence of resistance reservoirs among untreated HIV-infected persons has multiple implications. Antiretroviral therapy is increasingly successful at long-term suppression of viremia (31), with a consequent reduction in rates of transmitted resistance from these individuals (45). The persistence of resistant viruses in drug-naïve individuals suggests that there is a limit to the reduction in rates of transmitted HIV drug resistance. Current treatment guidelines recommend initiation of therapy when CD4 counts are below 350 cells per μl (14). This allows up to 30% of the diagnosed population (and all undiagnosed individuals) to maintain high viremia and infectivity. It is such individuals in whom circulation of resistance will continue. We argue that current discussions on an earlier start of therapy, such as the Strategic Timing of AntiRetroviral Treatment trial, should also consider the public health benefits of reducing transmission rates of drug-resistant viruses.

The cohort studied in the present work is, to our knowledge, the largest population of drug-naïve and -experienced HIV-1 patients from a single country that has been used for a molecular epidemiological analysis to date. Out of the 60,000 individuals diagnosed with HIV in the United Kingdom, about 55,000 HIV *pol* gene sequences were sequenced, a small fraction of which were longitudinally generated from the same patient. We therefore estimate that about 70% of known HIV-positive individuals in the United Kingdom are represented in our data set. Yet only 45 out of the 4,870 (~0.9%) drug-naïve subtype B viruses included were involved in transmission of drug resistance. This percentage is remarkably low considering the current prevalence of drug resistance among untreated patients (5 to 10%) and estimated levels of ongoing transmissions within the population (23). However, the database does not represent all infected individuals in the United Kingdom, let alone those remaining undiagnosed, and our results are likely to underestimate the burden of drug-resistant reservoirs. This may also explain why previous studies of transmission networks based on smaller data sets failed to characterize resistant lineages (23, 30). Since all treated patients carrying resistant viruses in the United Kingdom are included in our database, it is highly unlikely that viruses from drug-experienced patients not represented within our data set are present as intermediaries in the lineages shown. Furthermore, the short internode intervals characterizing the five clusters narrow down the likelihood of missing lineages in these transmission networks. In any case, we believe that the possible involvement of treatment-experienced individuals in transmission clusters does not challenge our findings, since the acquisition of drug resistance would predate their connection to the network. These individuals may also have transmitted the virus prior to therapy. It is nonetheless possible that the presence of treated individuals in the clusters (such as in cluster B) could "boost" the persistence of drug resistance in the untreated population. With about 80% of new HIV diagnosis patients undergoing resistance testing since 2007 in the United Kingdom, our sequence database will increasingly represent all diagnosed individuals in the country, and future work on the transmission of drug resistance among untreated individuals will yield more accurate estimations.

In summary, given the current decrease in resistance trans-

mitted from treated individuals, an increasing proportion of such transmissions is likely to derive from drug-naïve lineages. The extent to which reservoirs of resistance can persist in a drug-naïve population has yet to be estimated, but the very existence of these sustained reservoirs suggests there is a limit to the decline of transmitted drug resistance. These findings provide new insights for the planning and management of treatment programs in developing countries.

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